Supporting Information

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SI Discussion

With the development of the free fatty acids (FFA)-secretion strains, we found that an FFA strain cannot keep secreting forever, and that the secretion efficiency dropped when the culture became very old (after 1 mo). A significant amount of biomass was left after production. To make use of the spent biomass, we decided to utilize lipolyitic enzymes to hydrolyze the membrane lipids (diacylglycerol) into FFAs. For controlling the lipolytic genes, we considered metal inducible promoters, high light inducible promoters, heat shock inducible promoters, stationary growth phase inducible promoters, Fe-starvation-inducible promoters, N-starvation-inducible promoters, CO_2-enhancement-inducible promoters, and CO_2-limitation-inducible promoters. We decided to use the CO_2-limitation to induce the lipolytic enzymes because we consider it economic (without adding anything) and sensitive (CO_2 depleted in 15 min after stopping bubbling) for cyanobacteial culture grown in a photobioreactor.

To test the feasibility of this idea, we constructed two testing strains SD236 [Δ*nrsBAC*::P_{nrsB} *fol* ribosome binding site (RBS) *shl*] and SD237 (P_{cmp}::*fol* RBS *shl*). In SD236, two lipolytic genes were controlled by the proven nickel inducible control system to test the performance of lipolytic enzymes in cyanobacteria. Fortunately, the first Green Recovery strain SD237 worked (autolyzed) with CO_2 limitation. After the pilot strains, we made the second batch strains containing individual lipolytic genes (SD252, SD256, SD257, and SD258) to evaluate the independent performance of the lipolytic enzymes for Green Recovery. In the process of construction, we found the double cross-over efficiency of the P_{sbt} site is much lower than that of the P_{cmp} site, thus making the screening SD252 (P_{sbt} *gpl*) much harder than the other strains with insertion at the P_{cmp} site. We also combined the Green Recovery modifications with an FFA-secretion strain SD232 to make the third batch strains SD239 and SD262. Results showed that the self-destruction rate of Green Recovery was faster in the FFA-secretion background strains than the wild-type background strains.

Green Recovery is able to release other biofuel molecules retained inside the cells, such as triacylglycerols, alkanes, and fatty acyl esters (biodiesel). We believe the technique will significantly reduce the microalgal biofuel production cost, if Green Recovery is introduced into algae.

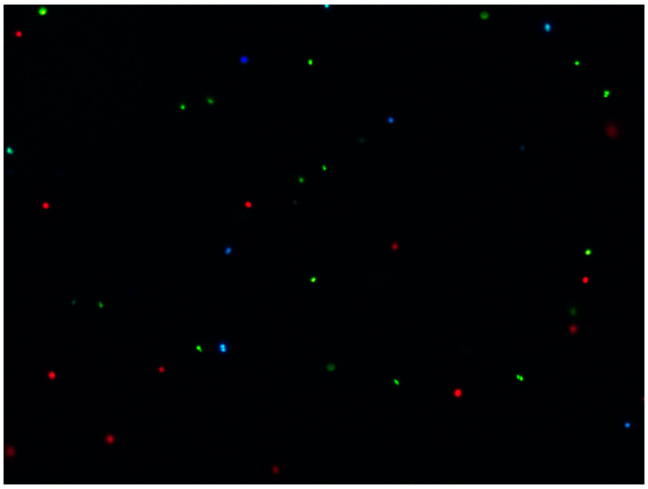

Fig. S1. One fluorescence microscopy picture of a SYTOX green stained SD256 culture 2 d after CO_2 limitation. Under fluorescence microscopy with 460–490 nm excitation, cells in a 6803 culture usually have three colors. Red cells are counted as membrane intact cells, where red is the autofluorescence of cyanobacterial phycobilisomes. Green cells are counted as membrane damaged cells, where green is the fluorescence of SYTOX green penetrating inside the cells and binding with DNA. Blue cells are also counted as damaged cells, which could be ghost cells with DNA and pigments already leaked out. For each 6803 sample, at least 200 cells were counted and analyzed for membrane permeability.

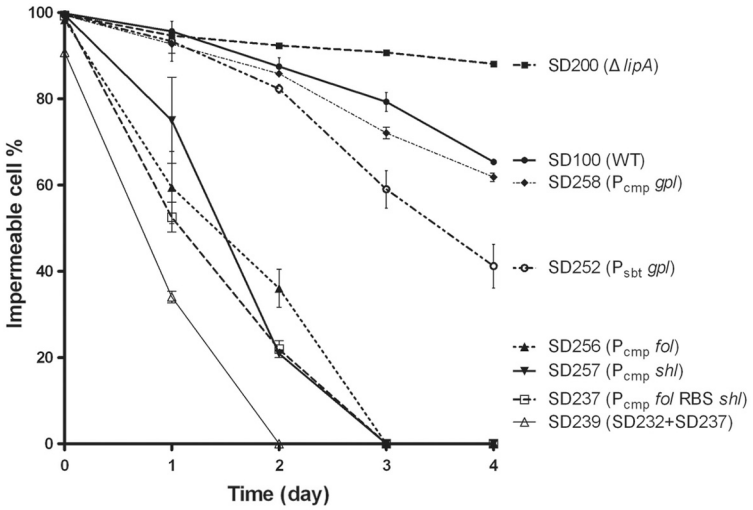

Fig. S2. Membrane damage after CO_2 limitation for different Sun Devil (SD) strains. The membrane damage was revealed by SYTOX staining. The starting cell densities and the estimated damage rates are listed in Table 1.

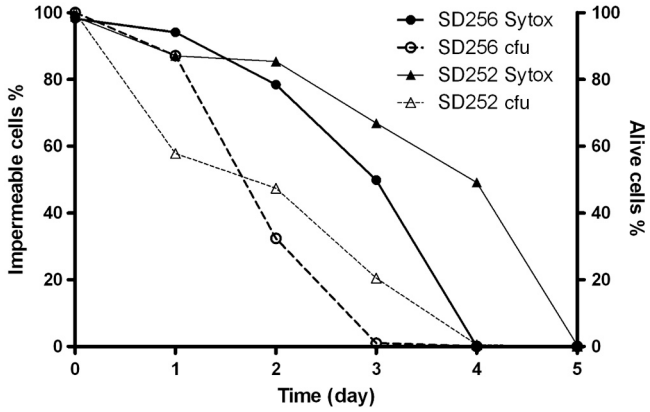

Fig. S3. The relationship between membrane permeability (revealed by SYTOX staining) and cell viability (revealed by cfu) during Green Recovery after CO_2 limitation.

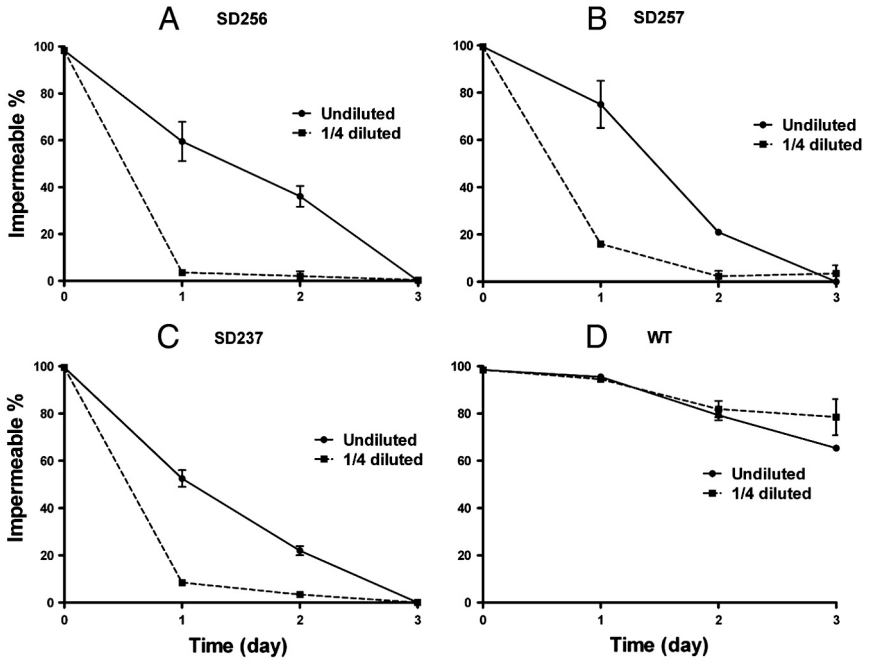

Fig. S4. The membrane damage of different SD strains after CO_2 limitation with one-fourth dilution of the original culture.

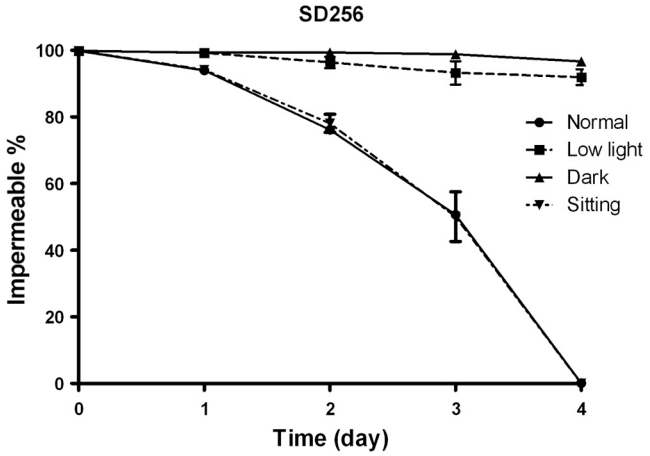

Fig. S5. The membrane damage of SD256 after CO_2 limitation under different conditions. Normal means the CO_2-limited cultures were rotated at 100 rpm under continuous illumination (140 μmol photons m^{-2} s^{-1}); low light means the CO_2-limited cultures were rotated at 100 rpm under illumination (20 μmol photons m^{-2} s^{-1}); dark means the CO_2-limited cultures were rotated at 100 rpm under illumination (2 μmol photons m^{-2} s^{-1}); sitting means the CO_2-limited cultures were shaken only once per day before sampling and under illumination (140 μmol photons m^{-2} s^{-1}).

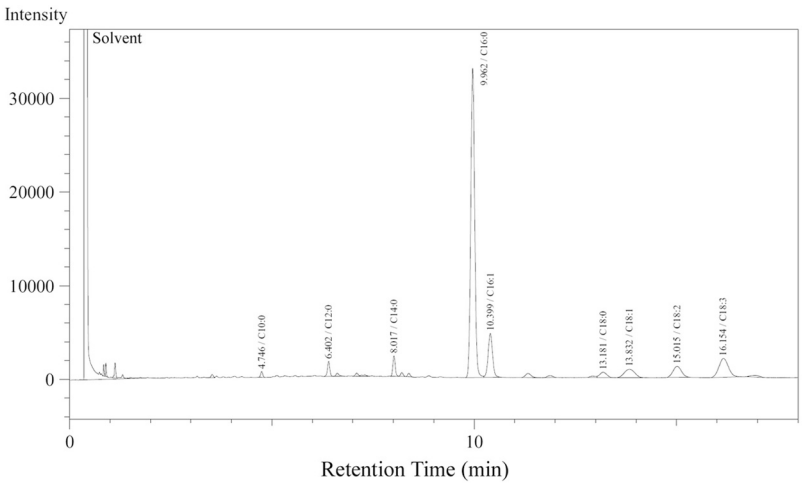

Fig. S6. The GC analysis of the FFA samples extracted by hexane from the SD237 culture after CO_2 limitation. The retention time and the types of released FFAs are marked on the peaks.

Table S1. SD strains constructed for this study

SD No.	Genotype *	Construction	Remarks
SD100	*Synechocystis* sp. PCC6803 wild-type		from Dr. Wim Vermaas's lab, School of Life Science, Arizona State University
SD200	Δ*lipA*::Km^R *sacB*	transform SD100 with pΨ200 (Δ*sll1969*::Km^R *sacB*) and select for kanamycin resistance	deletion of a putative 6803 lipase gene *sll1969*; from 908392 to 908941 in the chromosome (Cyanobase)
SD232	Δ*slr1609*::P_{psbA2} *'tesA* Δ(*slr1993-slr1994*)::P_{cpc} *accBC* P_{rbc} *accDA* Δ*sll1951*::*P_{psbA2} Uc *fatB1* P_{rbc} Ch *fatB2*	constructed in previous research	third generation constitutive FFA-secreting strain; plant (*Umbellularia californic* and *Cuphea hookeriana*) and *Escherichia coli* thioesterases and ACC were overproduced, and an acyl-ACP synthetase gene, PHB synthesis genes, and a surface layer gene were deleted in SD100
SD234	P_{cmp}::Km^R *sacB*	transform SD100 with pΨ234 (P_{cmp}::Km^R *sacB*) and select for kanamycin resistance	intermediate strain for inserting genes under the control of P_{cmp} on the basis of SD100
SD235	Δ*slr1609*::P_{psbA2} *'tesA* Δ(*slr1993-slr1994*)::P_{cpc} *accBC* P_{rbc} *accDA* Δ*sll1951*::*P_{psbA2} Uc *fatB1* P_{rbc} Ch *fatB2* P_{cmp}::Km^R *sacB*	transform SD232 with pΨ234 (P_{cmp}::Km^R *sacB*) and select for kanamycin resistance	intermediate strain for inserting genes under the control of P_{cmp} on the basis of SD232
SD237	P_{cmp}::*fol* RBS *shl*	transform SD234 with pΨ237 (P_{cmp}::*fol* RBS *shl*) and select for sucrose survival	Green Recovery strain with *fol* and *shl* controlled by P_{cmp}
SD239	Δ*slr1609*::P_{psbA2} *'tesA* Δ(*slr1993-slr1994*)::P_{cpc} *accBC* P_{rbc} *accDA* Δ*sll1951*::*P_{psbA2} Uc *fatB1* P_{rbc} Ch *fatB2* P_{cmp}::*fol* RBS *shl*	transform SD235 with pΨ237 (P_{cmp}::*fol* RBS *shl*) and select for sucrose survival	combination of SD232 and SD237, a FFA-secretion plus Green Recovery strain
SD244	P_{sbt}::Km^R *sacB*	transform SD100 with pΨ244 (P_{sbt}::Km^R *sacB*) and select for kanamycin resistance	intermediate strain for inserting genes under the control of P_{sbt} on the basis of SD100
SD245	P_{cmp}::*fol* RBS *shl* P_{sbt}::Km^R *sacB*	transform SD237 with pΨ244 (P_{sbt}::Km^R *sacB*) and select for kanamycin resistance	intermediate strain for inserting genes under the control of P_{sbt} on the basis of SD237
SD246	Δ*slr1609*::P_{psbA2} *'tesA* Δ(*slr1993-slr1994*)::P_{cpc} *accBC* P_{rbc} *accDA* Δ*sll1951*::*P_{psbA2} Uc *fatB1* P_{rbc} Ch *fatB2* P_{cmp}::*fol* RBS *shl* P_{sbt}::Km^R *sacB*	transform SD239 with pΨ244 (P_{sbt}::Km^R *sacB*) and select for kanamycin resistance	intermediate strain for inserting genes under the control of P_{sbt} on the basis of SD239
SD252	P_{sbt}::*gpl*	transform SD244 with pΨ252 (P_{sbt}::*gpl*) and select for sucrose survival	Green Recovery strain with *gpl* controlled by P_{sbt}
SD253	P_{cmp}::*fol* RBS *shl* P_{sbt}::*gpl*	transform SD245 with pΨ252 (P_{sbt}::*gpl*) and select for sucrose survival	combination of SD237 and SD252, with 3 lipolytic genes
SD254	Δ*slr1609*::P_{psbA2} *'tesA* Δ(*slr1993-slr1994*)::P_{cpc} *accBC* P_{rbc} *accDA* Δ*sll1951*::*P_{psbA2} Uc *fatB1* P_{rbc} Ch *fatB2* P_{cmp}::*fol* RBS *shl* P_{sbt}::*gpl*	transform SD246 with pΨ252 (P_{sbt}::*gpl*) and select for sucrose survival	combination of SD232, SD237 and SD252, with 3 lipolytic genes and FFA-secretion
SD256	P_{cmp}::*fol*	transform SD234 with pΨ256 (P_{cmp}::*fol*) and select for sucrose survival	Green Recovery strain with individual lipolytic enzyme Fol
SD257	P_{cmp}::*shl*	fransform SD234 with pΨ257 (P_{cmp}::*shl*) and select for sucrose survival	Green Recovery strain with individual lipolytic enzyme Shl

SD No.	Genotype *	Construction	Remarks
SD258	P_{cmp}::*gpl*	transform SD234 with pΨ258 (P_{cmp}::*gpl*) and select for sucrose survival	Green Recovery strain with individual lipolytic enzyme Gpl
SD262	Δ*slr1609*::P_{psbA2} *'tesA* Δ(*slr1993-slr1994*)::P_{cpc} *accBC* P_{rbc} *accDA* Δ*sll1951*::*P_{psbA2} Uc *fatB1* P_{rbc} Ch *fatB2* P_{cmp}::*fol* RBS *shl* P_{sbt}::*gpl* RBS *13 19 15*	transform SD246 with pΨ262 (P_{sbt}::*gpl* RBS *13 19 15*) and select for sucrose survival	on the basis of SD254, the *Salmonella* phage P22 lysis cassette *13 19 15* was added under the control of P_{sbt}

*Genetic information: *lipA* (*sll1969*), a putative lipase gene in 6803 chromosome; *sacB*, *sacB* gene, which is lethal for cyanobacteria in the presence of sucrose; Km^R, kanamycin resistance cassette; *slr1609*, the 6803 fatty acid activation gene, encoding an acyl–acyl carrier protein synthetase (1); P_{psbA2}, the promoter of 6803 *psbA2* gene, a 6803 strong promoter (2); *'tesA*, an *E. coli* thioesterase gene without the export signal sequence (3); *slr1993* and *slr1994*, two poly-3-hydroxybutyrate (PHB) synthesis genes; P_{cpc}, the promoter of 6803 *cpc* operon (4); P_{rbc}, the promoter of 6803 *rbc* operon (5); *accB*, *accC*, *accD*, and *accA*, four genes coding for 6803 acetyl-CoA carboxylase (ACC) subunits (6); *sll1951*, encoding a hemolysin-like protein, which is a 6803 surface structural protein (7); *P_{psbA2}, an improved promoter from P_{psbA2}; Uc *fatB1*, a thioesterase (TE) gene from *Umbellularia californica* (8); Ch *fatB2*, a TE gene from *Cuphea hookeriana* (9); P_{cmp}, the promoter of 6803 *cmp* operon; *fol*, a synthesized gene based on the amino acid sequence of the fungal phospholipase from *Fusarium oxysporum* (Fol); *shl*, a synthesized gene based on the amino acid sequence of the lipase from *Staphylococcus hyicus* (Shl); *gpl*, a synthesized gene based on the amino acid sequence of guinea pig pancreatic lipase-related protein 2; P_{sbt}, the promoter of 6803 *sbtA* gene; *13 19 15*, *Salmonella* phage P22 lysis cassette.

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9 Dehesh K, Jones A, Knutzon DS, Voelker TA (1996) Production of high levels of 8:0 and 10:0 fatty acids in transgenic canola by overexpression of Ch FatB2, a thioesterase cDNA from *Cuphea hookeriana*. *Plant J* 9:167–172.

Table S2. The recovered FFA fraction of total cellular fatty acids in Green Recovery

Green recovery strains	Initial density, 10^8 cfu/mL	Total FA in cells,* mg/L	Recovered FFA,† mg/L	Recovery rate, %	Fatty acid left in debris,‡ mg/L	Debris leftover rate, %
SD256	4.05	47.2 ± 0.4	19.1 ± 4.1	40.4	13.4 ± 1.0	28
P_{cmp47}::*fol*	1.01	11.8 ± 0.1	3.2 ± 0.1	27.1	4.4 ± 0.2	37
SD237	3.65	42.1 ± 0.8	19.0 ± 3.0	45.2	7.8 ± 0.2	19
P_{cmp43}::*fol* RBS *shl*	0.91	10.5 ± 0.2	3.6 ± 0.07	34	3.4 ± 0.8	32.4

*The total fatty acids in the cells (composed of membrane diacylglycerol lipids) were extracted by Folch method and transesterified into fatty acid methyl ester by methanolic HCl for GC measurement.
†The recovered FFA were extracted by hexane with acidification and also transesterified into fatty acid methyl ester for GC measurement.
‡The fatty acids left in the cell debris after hexane extraction were extracted by Folch method and transesterified into fatty acid methyl ester by methanolic HCl for GC measurement.

Table S3. Primers used in this study

Primer name	Sequence (5′ to 3′)
Construction of pΨ200	
LipA-S	TACCTGGGTTCAGGGTTGTGAT
LipA-A	CAAGCTTGTGCAACAGTCGGAA
Construction of pΨ234	
cmpR-s	TCT GGT TCC AGA GCC TGA TTC CCT CAT AAT CAA G
cmpA-a	ATC CGC TCG AAT GGC GAG GTA CTC TT
cmpR-a	TTC TAT GAA AAA AGG CAG ACA GAA AAA TTA AAT AAG CGC CA
cmpA-S	GAA AAA ATC AAT CAA AGT TAT GGG TTC ATT CAA TCG ACG
Construction of pΨ237, pΨ256, and pΨ257	
Fol-s	TTG AGG AGG TGT GAT GGA AGT ATC CCA AGA TCT GTT TAA T
Fol-a	CTT CAT TGA ATT AAT CTC CTC ATT AAC TGA AAC CGC CGG CGT TA
Shl-s	CAG TTA ATG AGG AGA TTA ATT CAA TGA AGC CTA CCG TTA AAG CTG
Shl-a	TCG ATA TCA TTA GGC GTT TTT GGT AGA TTC GGC TTT TTC G
Construction of pΨ244	
S8F1-S	GGT TCG ACA AAT TTG AGA TAG TTT TGT GGC AAA G
S8F1-A	T GTA AAT TGT CTC CTT GGT TGA AAT TTA TGG GGA
S8F2-S	AGG AGA ACA TTA TGG ATT TTT TGT CCA ATT TCT TGA CG
S8F2-A	CCA TAA TCA GCA TCA AGA TGG AAA GTA GTC CTC G
Construction of pΨ252 and pΨ258	
GPLRP2-S	TAT GAA ATT GTT TGC CTG GAC TAT TGG TTT ACT GCT G
GPLRP2-A	TTT AGC AGG GGC TCA GAG TCT GCT CGA CGT T
Construction of pΨ262	
P2213-S	AGG AGG ATA TAA TGC CAG AAA AAC ATG ATC TGT TAA CCG
P2215-A	aTTA TTT TAA GCA CTG ACT CCT GAT GTA CTC CTG CAG
GPL-END-FOR	AAG ACG GGA CCA AAT ATA ACT TCT GCT CCA GCG AT
P2213-BACK	ATG CCC TGT TCC TTT GCC GCC ATC ATG
Segregation checking/sequencing	
S6-66S	TTT ACC TAG CCT AGG AGC CCC AGT G
S6-99A	GCA CAA CCC TTC AGA AAT AAA GCC CCT
S8-90S	CTG GAA AGA TCA AGC AAA CTG CCG AAG ATT CA
S8-60A	ACT GCA ATT GTC CCA CGA AGT CCG TCA A

GPLRP, guinea pig pancreatic lipase-related protein 2.

Table S4. Synthesized DNA segments

Fol RBS *shl*

TTCCCAATTTGAGGaGGTGTG
ATG GAA GTa TCC CAa GAT CTG TTT AAT CAA TTC AAT CTG TTC GCT CAA TAC TCC GCg GCT
GCT TAC TGT GGg AAA AAT AAC GAT GCT CCT GCg GGc ACC AAT ATT ACt TGT ACC GGT AAC
GCT TGC CCT GAA GTG GAg AAg GCT GAC GCC ACC TTT TTG TAC AGC TTC GAA GAT AGC GGC
GTA GGC GAT GTG ACT GGt TTC cTc GCC TTG GAT AAT ACC AAT AAA TTG ATT GTT CTC TCT
TTC CGT GGC agt GAc TCC ATT GAG AAT TGG ATT GCC AAC TTG AAc TTt TGG TTG AAG AAA
ATC AAt GAt ATT TGT TCC GGc TGT CGG GGt CAC GAT GGT TTC ACC AGC AGC TGG CGT TCC
GTG GCC GAC ACC CTC CGG CAA AAG GTg GAa GAT GCT GTg CGc GAA CAt CCt GAT TAC CGC
GTT GTT TTT ACC GGG CAC TCC CTG GGC GGG GCC TTG GCC ACC GTA GCC GGT GCT GAC CTG
CGC GGT AAC GGC TAC GAT ATT GAT GTG TTC TCC TAT GGG GCT CCC CGG GTT GGG AAC CGG
GCT TTT GCt GAg TTT TTG ACC GTt CAa ACC GGc GGT ACC TTG TAT CGG ATC ACC CAT ACC
AAT GAT ATT GTT CCC CGC CTG CCC CCT CGT GAA TTC GGT TAT AGC CAC TCC TCC CCC GAA
TAC TGG ATT AAA AGC GGC ACC TTG GTT CCC GTG ACC CGT AAC GAC ATT GTA AAA ATT GAG
GGC ATT GAC GCC ACC GGT GGT AAT AAT CAA CCT AAT ATC CCC GAT ATC TTG GCT CAT CTG
TGG TAT TTT CAA GCC ACC GAC GCT TGT AAC GCC GGC GGT TTC AGT TAA
T**GAGGAGA**TTAATTCA
ATGAAGCCTACCGTTAAAGCTGCTCCCGAGGCTGTTCAGAACCCGGAAAACCCGAAAAA
CAAGGACCCCTTTGTGTTTGTGCACGGCTTTACCGGTTTTGTGGGGGAGGTTGCTGCG
AAAGGTGAGAATCACTGGGGCGGCACCAAAGCCAATCTGCGCAACCATTTGCGGAA
AGCTGGTTACGAAACCTACGAAGCCTCCGTATCCGCCTTGGCCTCCAATCACGAACG
TGCTGTGGAACTGTACTATTATCTGAAAGGTGGTCGGGTAGACTATGGTGCTGCCC
ATTCCGAAAAATATGGCCATGAGCGTTACGGGAAAACTTATGAAGGTGTGCTGAAAG
ATTGGAAACCCGGGCACCCCGTACACTTTATCGGTCATTCCATGGGTGGTCAGACCA
TTCGGCTGCTGGAACATTATCTGCGCTTTGGTGATAAAGCCGAAATTGCCTATCAAC
AACAGCACGGGGGTATTATTAGCGAATTATTTAAGGGCGGTCAAGACAACATGGTGA
CCTCTATCACTACTATTGCCACCCCTCACAATGGTACCCATGCTTCTGACGATATTG
GCAATACCCCGACTATCCGGAACATTCTGTATAGCTTCGCCCAAATGTCCAGTCATC
TGGGCACCATCGACTTTGGGATGGACCATTGGGGTTTCAAGCGGAAAGATGGCGAG
AGTCTGACCGATTATAATAAGCGGATTGCC
GAGAGCAAAATCTGGGATTCTGAAGATACTGGGCTGTATGACCTGACCCGTGAAGGC
GCCGAGAAAATCAACCAGAAAACCGAATTGAATCCCAATATCTATTACAAAACCTAC
ACTGGGGTGGCTACCCATGAAACTCAGTTAGGCAAACACATCGCGGACCTCGGCATG
GAATTTACCAAAATCCTCACCGGCAACTATATCGGGAGCGTAGACGATATTCTGTG
GCGGCCCAATGATGGTTTGGTGAGCGAAATCTCCAGCCAACACCCCAGCGATGAG
AAGAACATTTCCGTAGACGAAAACTCCGAACTGCATAAGGGTACCTGGCAGGTCATG
CCTACCATGAAAGGGTGGGACCACTCCGATTTTATTGGTAATGACGCCCTGGATACC
AAACACTCCGCCATCGAACTCACCAACTTTTATCATAGCATTTCTGACTACTTGATGC
GGATCGAAAAAGCCGAATCTACCAAAAACGCCTAATGATATCGA

gpl

ATG Aaa TTG TTT GCC TGG ACT ATT GGT TTA CTG CTg ctg GCC ACt GTG CGC GGc GCT GAG
GTc TGT TAT TCT CAt TTG GGC TGc TTT TCC GAC GAG AAA CCG TGG GCg GGT ACC TCT CAA
CGG CCC ATC AAg AGT TTG CCg TCC GAC CCT AAA AAG ATT AAt ACC CGG TTC TTG CTG TAC
ACC AAt GAa AAt CAA AAT TCC TAC CAA CTG ATC ACC GCT ACT GAT ATT GCC ACC ATC AAA
GCC AGT AAC TTC AAT CTc AAC CGc AAA ACC CGC TTC ATT ATT CAC GGT TTC ACC GAC AGC
GGT GAG AAC TCT TGG CTG AGT GAT ATG TGT AAA AAC ATG TTC CAA GTT GAA AAA GTG AAT
TGC ATT TGC GTG GAT TGG AAA GGC GGT TCC AAG GCT CAA TAC AGT CAG GCT TCC CAG AAT
ATT CGG GTG GTC GGT GCC GAA GTT GCC TAT TTA GTG CAA GTA CTG AGC ACC TCC CTG AAC
TAT GCC CCG GAA AAC GTA CAT ATT ATT GGT CAC TCC CTC GGC GCC CAC ACC GCT GGG GAA
GCC GGG AAG CGG CTG AAC GGG CTG GTA GGG CGG ATT ACC GGc cTc GAC CCC GCC GAA CCC
TAC TTT CAA GAT ACC CCC GAA GAA GTC CGG CTG GAT CCC TCT GAC GCT AAA TTT GTG GAC
GTG ATT CAC ACT GAT ATT AGC CCC ATT CTG CCT AGT CTG GGT TTC GGT ATG TCC CAG AAG
GTC GGT CAC ATG GAC TTC TTC CCC AAC GGC GGC AAA GAT ATG CCC GGG TGC AAA ACC GGC
ATC TCC TGC AAC CAC CAC CGG AGT ATT GAA TAT TAT CAC AGC AGT ATT TTG AAC CCC GAA
GGT TTt TTa GGC TAC CCG TGT GCT TCC TAC GAT GAA TTc CAA GAA TCt GGG TGC TTC CCC
TGT CCC GCT AAA GGT TGT CCT AAA ATG GGT CAC TTT GCC GAC CAG TAC CCC GGg AAG ACC
AAT GCT GTC GAA CAA ACC TTc TTC CTC AAC ACC GGg GCg TCC GAT AAT TTC ACC CGG TGG
CGC TAT AAA GTA ACC GTT ACC CTG AGT GGC GAA AAA GAC CCG TCC GGT AAC ATC AAT GTG
GCT TTG TTA GGT AAA AAC GGG AAC TCT GCT CAA TAC CAG GTT TTC AAG GGC ACC CTG AAG
CCC GAC GCC TCC TAT ACT AAT TCC ATT GAT GTC GAA CTC AAC GTT GGG ACC ATT CAA AAA
GTG ACC TTC CTG TGG AAA CGG AGC GGG ATC TCC GTC TCT AAA CCC AAG ATG GGT GCT TCt
CGc ATT ACt GTT CAA AGT GGT AAA GAC GGG ACC AAA TAT AAC TTC TGC TCC AGC GAT ATT
GTG CAA GAA AAC GTC GAG CAG ACT CTG AGC CCC TGC taa